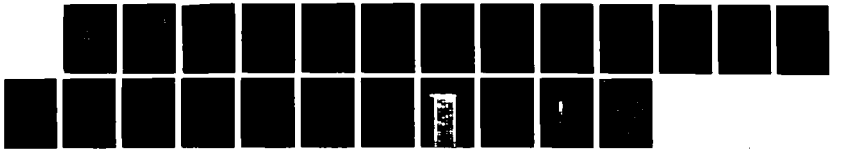
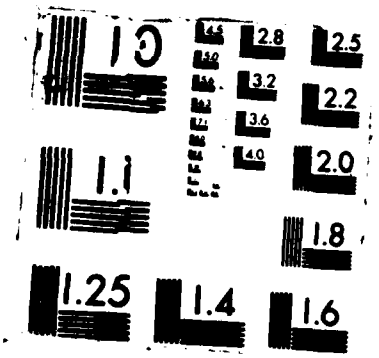
AD-A183 190 DESIGN AND SYNTHESIS OF SEMISYNTHETIC ENZYMES(U) 1/1
CALIFORNIA UNIV BERKELEY DEPT OF CHEMISTRY P G SCHULTZ
28 JUL 87 N00014-86-K-0522

UNCLASSIFIED F/G 6/1 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0

mnp FILE COPY

SECURITY CLASSIFICATION OF

AD-A183 190

TION PAGE

1a. REPORT SECURITY CLASSIF	RICTIVE MARKINGS NA
2a. SECURITY CLASSIFICATION AUTHORITY NA	3. DISTRIBUTION / AVAILABILITY OF REPORT Unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE NA	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) University of California, Berkeley	5. MONITORING ORGANIZATION REPORT NUMBER(S) NA
6a. NAME OF PERFORMING ORGANIZATION: Univ. California, Berkeley	6b. OFFICE SYMBOL (If applicable): NA
7a. NAME OF MONITORING ORGANIZATION: ONR	
6c. ADDRESS (City, State, and ZIP Code): Dept. of Chemistry, University of California, Berkeley, CA 94720	7b. ADDRESS (City, State, and ZIP Code): 800 N. Quincy St., Arlington, VA 22217-5000
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: ONR	8b. OFFICE SYMBOL (If applicable): ONR
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-86-K-0522	
8c. ADDRESS (City, State, and ZIP Code): 800 N. Quincy St., Arlington, VA 22217-5000	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
61153N	RR04106	441g012	

11. TITLE (Include Security Classification)

Design and Synthesis of Semisynthetic Enzymes

12. PERSONAL AUTHOR(S)

Peter G. Schultz

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 8/86 TO 7/87	7/28	20

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
08			Semisynthetic enzymes, unnatural amino acids nonsense suppression

Abstract

We propose to develop methodology which will for the first time enable specific amino acid residues in proteins to be site-specifically replaced with synthetic amino acid analogues. Amino acids with novel steric, electronic, or spectroscopic properties will then be used to probe structure-function relationships in proteins which lead to receptor/substrate recognition and/or catalysis.

We are pursuing a combined chemical/genetic strategy toward this objective. Our approach requires replacement of the codon for the target amino acid with an amber nonsense codon (5'-TAG-3') *via* oligonucleotide-directed mutagenesis. A nonsense suppressor tRNA that recognizes this unique codon is being constructed by both anticodon loop replacement and by chemical synthesis and overproduction (*E. coli*) of an amber suppressor gene, using known chemical/ biological procedures. This suppressor tRNA will then be chemically aminoacylated with novel amino acids by existing chemical methods, thereby avoiding the natural specificity of the aminoacyl tRNA synthetases. The aminoacylated tRNA will be used as a delivery vehicle *in vitro* for placing novel synthetic amino acids site-specifically into proteins. The methodology described above will be developed and optimized with the enzyme, β-lactamase, which catalyzes the hydrolysis of the β-lactam antibiotics penicillin and cephalosporin. Active site amino acids will be replaced with a series of synthetic amino acids and the physical and catalytic properties of these new semisynthetic enzymes will be characterized. We will then go on to examine structure-function relationships in a large number of enzymes of interest including staphylococcal nuclease, mercuric ion reductase, and aspartate-amino transferase.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	U

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Keith Ward	202-696-4760	ONR

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

DTIC ELECTE AUG 10 1987 S A

OFFICE OF NAVAL RESEARCH

END-OF-THE -YEAR REPORT

PUBLICATIONS/PATENTS/PRESENTATIONS/HONORS/STUDENTS REPORT

for

Contract N00014-86-K-0522

Code _____

Semisynthetic Enzymes

Peter G. Schultz

Department of Chemistry

University of California

Berkeley, CA 94720

Part I

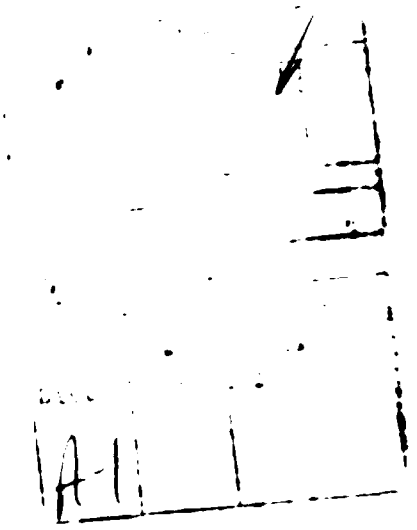
Honors/Awards/Prizes

Eli Lilly Scholar

Promoted to Associate Professor with tenure, 1987, University of California, Berkeley

Number of Graduate Students Receiving full or Partial Support 3

Number of Postdoctoral Fellows Receiving Full or Partial Support 1



Part II

Prof. Peter G. Schultz Principal Investigator

Dr. Keith Ward ONR Scientific Officer

Phone

Abstract

We propose to develop methodology which will for the first time enable specific amino acid residues in proteins to be site-specifically replaced with synthetic amino acid analogues. Amino acids with novel steric, electronic, or spectroscopic properties will then be used to probe structure-function relationships in proteins which lead to receptor/substrate recognition and/or catalysis.

We are pursuing a combined chemical/genetic strategy toward this objective. Our approach requires replacement of the codon for the target amino acid with an amber nonsense codon (5'-TAG-3) *via* oligonucleotide-directed mutagenesis. A nonsense suppressor tRNA that recognizes this unique "blank" codon is being constructed by both anticodon loop replacement and by chemical synthesis and overproduction (*E. coli*) of an amber suppressor gene, using known chemical/biological procedures. This suppressor tRNA will then be chemically aminoacylated with novel amino acids by existing chemical methods, thereby avoiding the natural specificity of the aminoacyl tRNA synthetases. The aminoacylated tRNA will be used as a delivery vehicle *in vitro* for placing novel synthetic amino acids site-specifically into proteins. The methodology described above will be developed and optimized with the enzyme, β-lactamase, which catalyzes the hydrolysis of the β-lactam antibiotics penicillin and cephalosporin. Active site amino acids will be replaced with a series of synthetic amino acids and the physical and catalytic properties of these new semisynthetic enzymes will be characterized. We will then go on to examine structure-function relationships in a large number of enzymes of interest including staphylococcal nuclease, mercuric ion reductase, and aspartate amino transferase.

Significant Results During Year

We have generated the required mutants of the bla gene with nonsense codons at positions 70 and 66 and expressed these genes behind efficient transcriptional promoters. The requisite suppressor

$tRNA^{Phe}_{CUA}$ has been constructed and progress has been made toward the synthesis and expression of a $tRNA^{Gly}_{CUA}$ in *E. coli*. Substantial progress has also been made toward developing efficient chemical aminoacylation protocols. Importantly, we have demonstrated that the methodology we are developing is capable of efficiently incorporating natural amino acids site selectively into proteins. We feel this is a strong indication that the methodology will incorporate unnatural amino acids as well.

Summary of Plans for Year 2

We plan to generate and fully characterize a series of mutant β-lactamases using enzymatically aminoacylated tRNA's and tRNA's misacylated with noncognate and possibly unnatural amino acids. This work will demonstrate the feasibility of the methodology. At the same time we will complete the chemical aminoacylation chemistry. The suppressor tRNA will then be chemically amino-acylated with synthetic unnatural amino acids and mutant enzymes will be generated and characterized. We also hope to express the $tRNA^{Gly}_{CUA}$ *gene, purify the tRNA and assay its ability to suppress* nonsense codons and its ability to be proofread.

Graduate Students and Postdoctoral Fellows

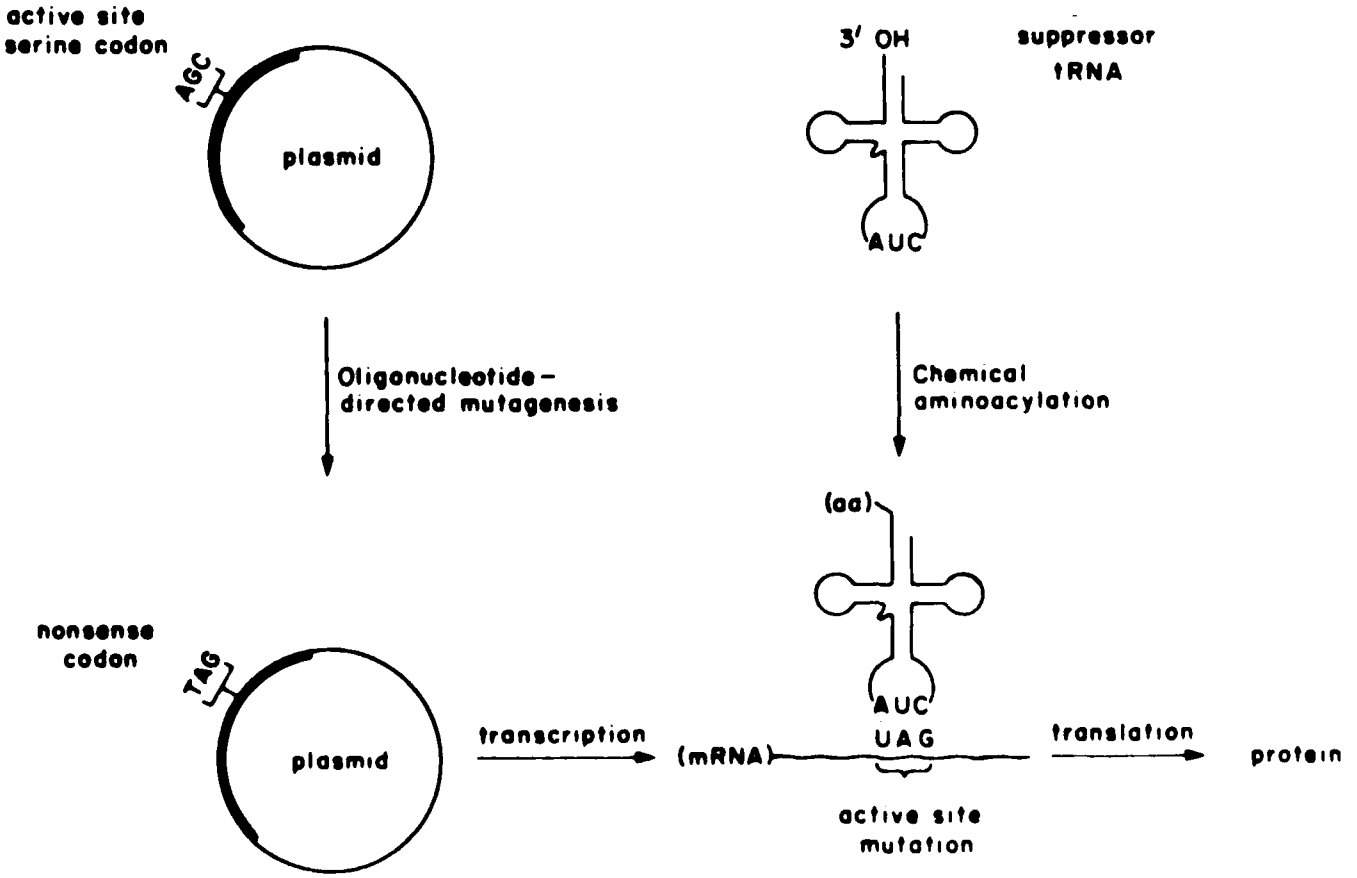
Spencer Anthony-Cahill
Christopher Noren
Michael Griffith
Dr. Ashis Saha

Technical Report

Oligonucleotide-directed mutagenesis is becoming a widely used technique for studying the relationship between primary structure and biological function in proteins. Site-directed mutagenesis has been used in conjunction with high resolution X-ray crystallography to measure contributions of enzyme active site residues to binding and catalysis as well as to produce new proteins with novel properties. In the absence of detailed structural information, site-directed mutagenesis has been used to probe for

essential catalytic residues. To date, however, only a limited number of substitutions (restricted to the natural 20 amino acids) can be used to probe a particular structural or electronic property of an amino acid at a specific site. The next logical step in this direction is to site-specifically substitute into proteins amino acids with unique structural, electronic, or spectroscopic properties tailored to probe the specific structure-function relationship at hand. The ability to rationally replace active site amino acids with a wide variety of structural variants would provide us with a more detailed picture of the mechanism of protein-ligand binding and catalysis.

We have devised a combined chemical/genetic approach toward this goal. Our strategy is to replace an active/binding site amino acid codon with a nonsense codon (differing from the gene's termination codon) *via* oligonucleotide-directed mutagenesis. A suppressor tRNA, directed against this nonsense codon, will then be chemically acylated with the desired amino acid and added to an *in vitro* transcription/translation system programmed with the mutagenized DNA. This system should direct insertion of the prescribed amino acid into the protein at the site corresponding to the location of the nonsense codon.



We have begun to develop the above methodology using the active site serine and phenylalanine of the enzyme β-lactamase as the initial targets for mutagenesis. β-lactamase catalyzes the hydrolysis of the amide bond of the lactam ring of penicillins and related antibiotics and is the most common cause of resistance to β-lactam antibiotics.

The sequence of the investigation is:

1) Oligonucleotide-directed mutagenesis of the active site serine 70 and phenylalanine 66 codons in the gene (bla) encoding β-lactamase to the amber nonsense codon, TAG, and expression of native and mutant bla genes both with and without the leader sequence behind the T7 and tac polymerase promoter;

2) Generation of the suppressor tRNA: (a) by anticodon loop replacement *via* chemical synthesis of the tetraribonucleotide 5'-CUAA-3' and chemical/enzymatic subsitution of CUAA into the anticodon loop of yeast $tRNA^{Phe}$ and (b) construction of a $tRNA^{Gly}_{CUA}$ *gene by automated solid phase synthesis and overproduction in E. coli.*;

3) Chemical aminoacylation of the suppressor tRNA by synthesis and 3'-OH acylation of the dinucleotide 5'-pCpA-3' with ^{14}C-serine or ^{14}C-phenylalanine followed by ligation into the acceptor stem of the nonsense suppressor tRNA;

4) Synthesis of β–lactamase in an *in vitro* protein synthesizing system in the presence of the chemically aminoacylated suppressor tRNA;

5) Optimization of each phase of the protocol and extension of the methodology to novel amino acids and other enzymes of interest.

Mutagenesis and Expression

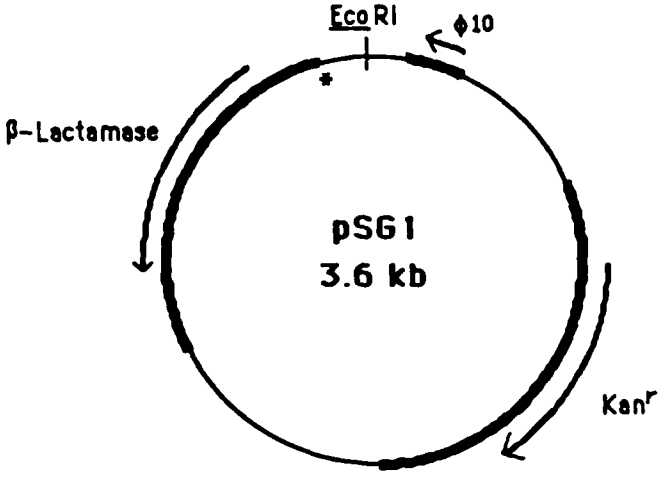
We have constructed and expressed the mutant β-lactamase genes in which the codons for both serine 70 and phenylalanine 66 have been replaced by the amber nonsense codon TAG. These

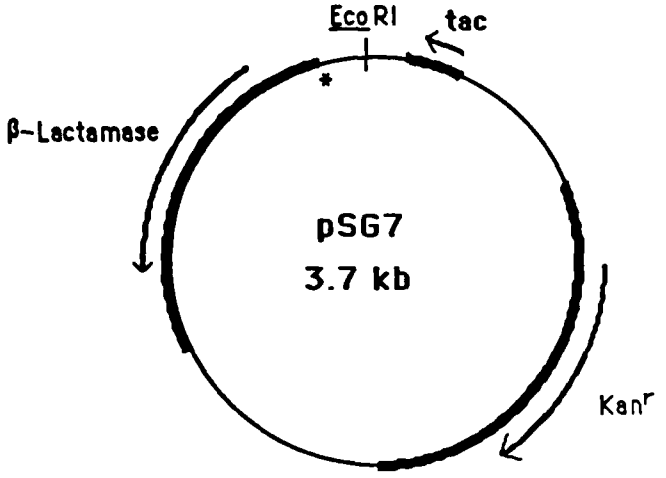
plasmids will enable us to replace either Ser 70 or Phe 66 with unnatural amino acids.

The serine 70 codon, AGC, of the gene encoding β-lactamase has been replaced with the nonsense codon TAG by oligonucleotide directed mutagenesis using the method of Zoller and Smith. The mutant and native genes were then placed behind both the T7 ϕ10 promoter and the hybrid tac promoter for efficient gene expression.

We have also constructed plasmids in which the region encoding the leader sequence of β-lactamase has been deleted so that *in vitro* DNA directed synthesis of β-lactamase results in the formation of fully processed enzyme.

More recently we have constructed mutants in which Phe66, which is conserved in all type I β-lactamases sequenced to date, was altered to the amber nonsense codon TAG, to TAT (Tyr) and to GCT (Ala). These mutants were generated by oligonucleotide mutagenesis using the method of Eckstein and coworkers. Again these mutant genes (lacking the region encoding the leader sequence) were placed begin the hybrid tac promoter. All mutant plasmids were characterized by dideoxy DNA sequencing and by restriction mapping.





* Denotes deletion of 63 bp corresponding to leader sequence.

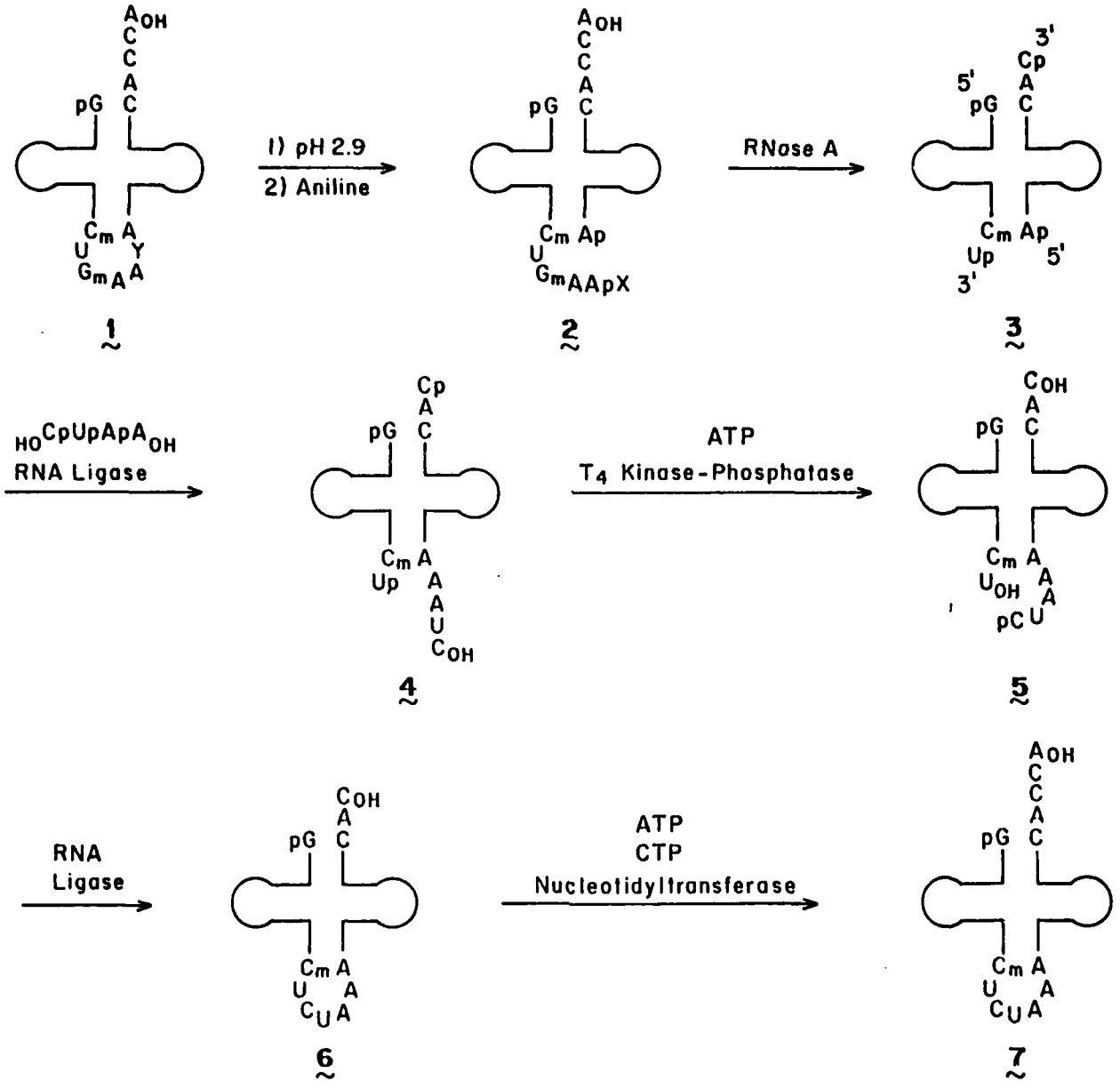
β-Lactamase mutants

	65	66	67	68	69	70	71
Wild-type sequence:	*Arg*	*-Phe*	*-Pro*	*-Met*	*-Met*	*-Ser*	*-Thr*
	-CGT-	TTT-	CCA-	ATG-	ATG-	AGC-	ACT-
Mutant 1 (pSG8):	*Arg*	*-Phe*	*-Pro*	*-Met*	*-Met*	*-Stop*	*-Thr*
	-CGT-	TTT-	CCA-	ATG-	ATG-	TAG-	ACT-
Mutant 2 (pSG9):	*Arg*	*-Tyr*	*-Pro*	*-Met*	*-Met*	*-Ser*	*-Thr*
	-CGT-	TAT-	CCA-	ATG-	ATG-	AGC-	ACT-
Mutant 3 (pSG10):	*Arg*	*-Ala*	*-Pro*	*-Met*	*-Met*	*-Ser*	*-Thr*
	-CGT-	GCT-	CCA-	ATG-	ATG-	AGC-	ACT-
Mutant 4 (pSG11):	*Arg*	*-Stop*	*-Pro*	*-Met*	*-Met*	*-Ser*	*-Thr*
	-CGT-	TAG-	CCA-	ATG-	ATG-	AGC-	ACT-

Construction of an Amber Nonsense Suppressor tRNA

We have completed the construction of a yeast amber $tRNA^{Phe}_{CUA}$ by the protocol of Uhlenbeck and coworkers. Yeast $tRNA^{Phe}_{CUA}$ was chosen as the target suppressor since work in the literature suggests that $tRNA^{Phe}_{CUA}$ will not be recognized by the *E. coli.* aminoacyl tRNA synthetases (in an *E. coli* coupled transcription/translation system) and will therefore not be proofread or reacylated. This requirement is essential to insure that homogeneous mutant proteins are synthesized by our methodology. The scheme for the construction of the suppressor tRNA, which is outlined below, required the synthesis of the tetraribonucleotide CUAA which is used to replace nucleotides 34-37 of yeast $tRNA^{Phe}$.

Construction of Suppressor tRNA



The tetranucleotide was synthesized from 2'methoxytetrahydropyranylated and base protected nucleoside monomers by the methods of Reese and of Van Boom. The fully protected nucleosides were 3'-phosphorylated and sequentially condensed with dihydroxynucleosides using mesitylenesulfonyl nitrotetrazole. The protected tetramer was deblocked by treatment with syn-pyridine aldoximate and concentrated ammonia and purified by chromatography on DEAE 25. The partially deblocked tetramer was then treated with 0.01 M HCl and purified by reverse phase HPLC (CH_3CN/0.01 M TEAB, pH 7.5) to yield the tetraribonucleotide CpUpApA. The final product was characterized by ^{31}P NMR, 500 Mhz ^{1}H NMR and by Fab mass spectrometry. We have approximately 5 g of the fully-protected tetraribonucleotide in hand.

The suppressor $tRNA^{Phe}_{CUA}$ was constructed by the method of anticodon loop replacement outlined above. The first step in the anticodon loop replacement protocol was cleavage of yeast $tRNA^{Phe}$ between nucleotides 37 and 38. We have purified large quantities (300 mg) of $tRNA^{Phe}$ from brewer's yeast. The hypermodified base, wyosine-37, was depurinated at pH 2.9 (37° C) to afford $tRNA^{Phe}$ which was purified from unreacted $tRNA^{Phe}$ by benzoylated (BD) cellulose chromatography. Cleavage was verified by denaturing polyacrylamide gel electrophoresis. The cleaved tRNA was then partially digested with ribonuclease at 0° C. This treatment resulted in removal of anticodon, G_mAA, and cleavage at C-74, removing the terminal dinucleotide CA from the acceptor step (this CA is replaced in the chemical aminoacylation step). Denaturing polyacrylamide gel electrophoresis confirmed the presence of the two-half molecules 133 and 38-74.

We have ligated the tetramer, CUAA, to the 5' terminal phosphate of the 3'-half molecule with T4 RNA ligase. We have in hand large quantities of purified T4 RNA ligase from *E. coli* strain E/KR5A containing a derivative of plasmid pDR54 in which the gene encoding T4 RNA ligase is under tac promoter control.

The construction of $tRNA^{Phe}_{CUA}$(-CA) was completed by dephosphorylating the 3'-phosphate and phosphorylating the 5'-hydroxyl of the tetranucleotide with T4 kinase and ligating the two half-molecules with T4 RNA ligase. The overall yield for production of $tRNA^{Phe}_{CUA}$ (-CA) was 27%. We have produced 24 mg of suppressor (lacking the 3' pCpA) in this fashion. In addition we have purified

large quantities of nucleotidyl transferase and T4 kinase required in the construction of the tRNA.

The 3' terminus was reconstructed using nucleotidyl transferase as described by Cudny and Deutscher. The reaction mixture contained ^{14}C-ATP. The reaction products were separated by gel electrophoresis (8% PAGE) and the radioactive band was eluted to yield the fully reconstructed suppressor tRNA (80% recovery). All intermediates in the construction of the suppressor tRNA were characterized by polyacrylamide gel electrophoresis and the final tRNA was sequenced to verify its structure. (see Appendix)

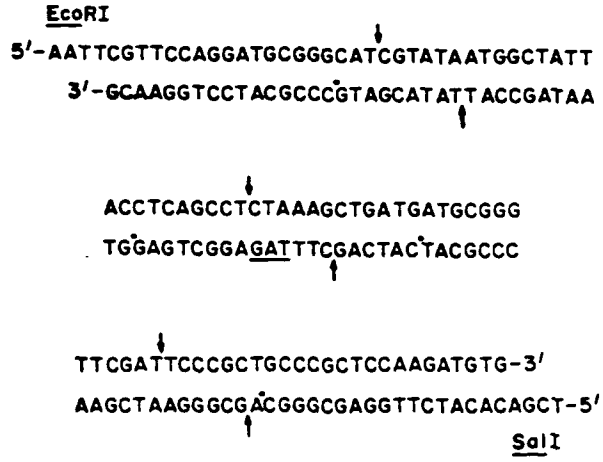
We have been able to aminoacylate the suppressor with phenylalanine using yeast phenylalanine tRNA synthetase which was purified by the method of Reid and coworkers. The lower limit for aminoacylation of the suppressor (based on liquid scintillation counting of TCA precipitated material) is 21%. Using similar reaction conditions wild-type yeast $tRNA^{Phe}$ is aminoacylated to at lest 42% of its amino acid acceptance capacity. The aminoacylated tRNA is purified by phenol extraction followed by ethanol precipatation prior to its addition to the *in vitro* protein synthesizing system.

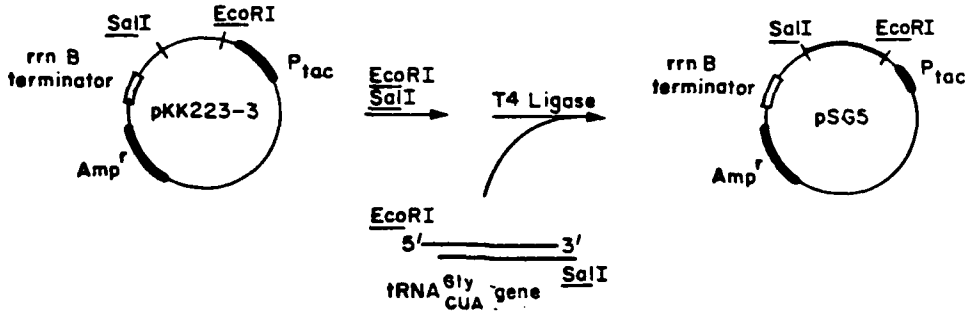
We have been unable to misaminoacylate the $tRNA^{Phe}_{CUA}$ using serine aminoacyl synthetase (yeast) under a variety of conditions including saturating enzyme, use of organic solvents, high pH and varying salt concentrations.

We have also initiated work aimed at constructing an overproducer of an *E. coli* $tRNA^{Gly}_{CUA}$. Our strategy is to replace the anticodon of $tRNA^{Gly}$ with CUA and express the resulting suppressor tRNA behind the strong tac transcriptional promoter. This strategy should afford large quantities of an amber nonsense suppressor tRNA which is not a substrate for *E. coli* tRNA synthetases (providing us with large readily obtainable supplies of suppressor tRNA's for future work).

The tRNA gene and flanking sequences were synthesized by solid phase DNA phosphoramidite synthesis. The gene was constructed from four overlapping oligonucleotides approximately 50-60 nucleotides in length. The gene was assembled by ligation of the annealed phosphorylated oligonucleotides using T4 DNA ligase in the presence of EcoRI/SalI restricted M13mp18 and EcoRI/SalI restricted M13mp19. This protocol afforded the fully assembled gene cloned into the polylinker sites of the single-stranded phage DNAs for sequencing. We have confirmed the sequence of the entire gene by sequencing of the gene in both directions. The EcoRI/SalI fragment of M13mp18, containing the $tRNA^{Gly}_{CUA}$ gene was subcloned

fragment of M13mp18, containing the $tRNA^{Gly}_{CUA}$ gene was subcloned into the expression vector pKK223-3. This plasmid contains the hybrid tac promoter to the 5' side of the tRNA gene and the transcriptional terminator rrnB to the 3' side of the tRNA gene. We are currently attempting to overproduce and purify the suppressor tRNA from IPTG induced *E. coli.* JM101 transformed with the gene.





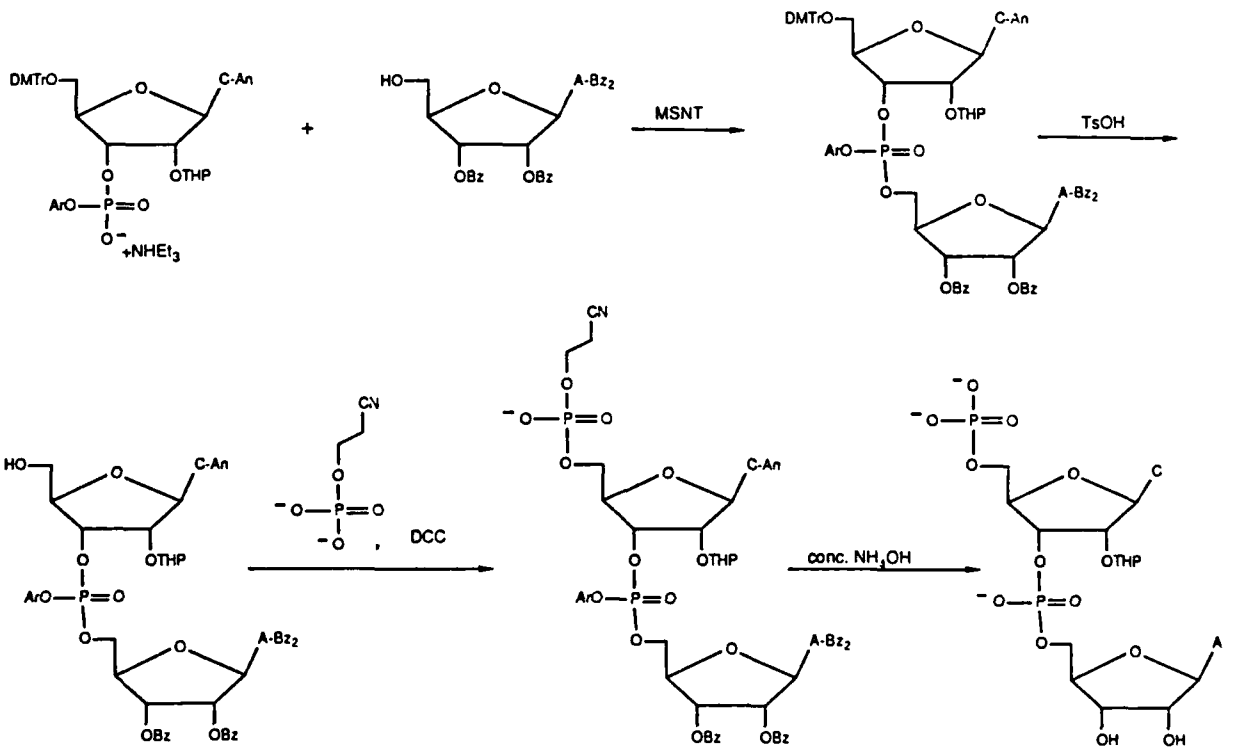
(III) Aminoacylation of $tRNA^{Phe}_{CUA}$

We are in the process of aminoacylating $tRNA^{Phe}_{CUA}$ (-CA) using a variation of the method described by Hecht. The aminoacylated tRNA is constructed by aminoacylating pCpA and ligating the 2'(3')-

0-aminoacyl-pCpA dinucleotide to the acceptor stem of $tRNA^{Phe}_{CUA}$(-CA) with T4 RNA ligase. This strategy avoids extensive protection of the entire tRNA. Note that we have considerable quantities of tRNA (-CA) on hand as it is an intermediate in the synthesis of the suppressor tRNA.

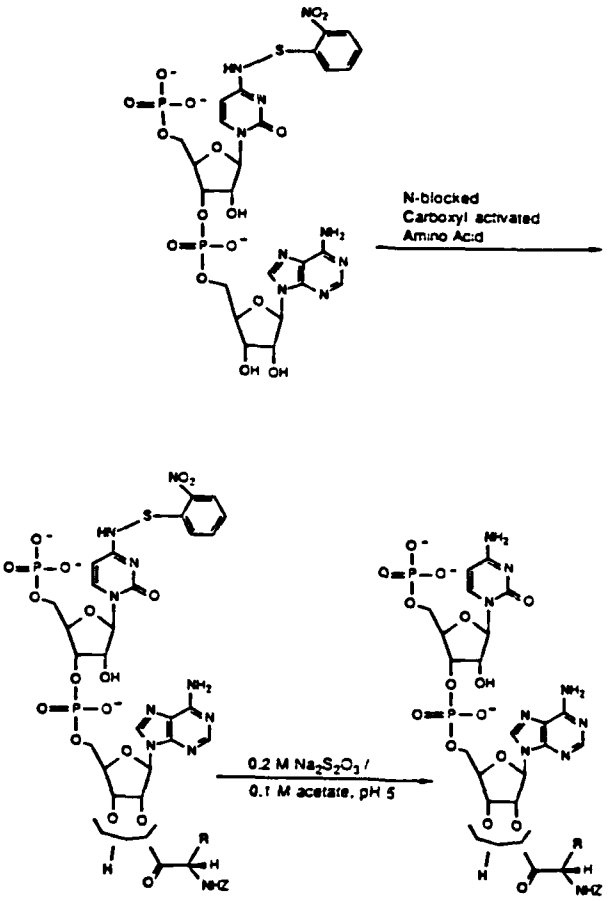
We have synthesized gram quantities of protected pCpA by the route outlined below and have purified the fully deprotected dinucleotide to carry out aminoacylation studies.

Synthesis of pCpA



Because the aminoacylation chemistry described in the literature results in a low yield of aminoacylated pCpA we have begun to develop new aminoacylation chemistry as outlined below.

The exocyclic amine of cytidine will be protected by the o-nitrophenylsulfenyl group which can be removed under mild reducing conditions. The α- amino group of the amino acid will be protected with a Cbz or CbzPhe protecting group which can be removed by hydrogenolysis or proteolysis, respectively, without affecting hydrolysis of the 2'(3') acyl group.



We have carried out preliminary experiments with 5' dimethoxytrityl cytidine in which the exocyclic amino group is protected with the o-nitrophenylsulfenyl group. Cbz protected alanine and phenylalanine have been coupled to the protected nucleoside in greater than 40% isolated yield using a 6 fold excess of N-protected amino acid in DMSO at room temperature with carbonyldiimidazole. We are in the process of deprotecting the NPS group and assaying the stability of the N-blocked and free α-amino aminoacylated nucleoside. We have also protected the exocyclic

labile to light or hydrogenation. Finally we have verified that pCpA can be coupled to $tRNA^{Phe}_{CUA}$ (-CA) in high yield using RNA ligase.

In Vitro Transcription/Translation

The *in vitro* transcription/translation system of Zubay, as modified by Collins and Pratt, has been successfully used to express functionally active RTEM β-lactamase from the truncated β-lactamase gene, which lacks the region coding for the leader sequence.

The gene has been expressed under the transcriptional control of both the bacteriophage T7 ϕ 10 promoter and the hybrid *E. coli* tac promoter. In former cases, the standard 30 μL reaction mixture is supplemented with 200 units of T7 RNA polymerase, whereas no exogenous enzymes are added in the latter case. Optimized reaction mixture (30 μL) contain, in addition to plasmid DNA (3 μg), the four nucleotide triphosphates (0.85 mM CTP, UTP, and GTP, 1.2 mM ATP), the twenty amino acids (0.35 mM each), dithiothreitol (1.8 mM), phosphoenol pyruvate (27 mM), folic acid (34.6 μg/ml), glycol 6000 (2%), *E. coli* tRNA (0.17 mg/mL), ammonium acetate (36 mM), potassium acetate (72 mM), and calcium acetate (9.7 mM) in a tris-acatate buffer (60 mM, pH 8.2) Protein synthesis is initiated upon the addition of 8.5 μL of S-30 cell extract containing pyruvate kinase (2 units/mL). Typical yields of β-lactamase are 20 and 35 nitrocefin units/mL/h for the T7 and tac systems, respectively. Based on the known specific activity of β-lactamase produced *in vivo* this corresponds to 25-30 copies of enzyme (per copy of gene per ~30 to 40 μg enzyme/mL) for the tac system.

We have purified β-lactamase produced *in vitro* to homogeneity in 21% overall yield (6 units from a 1 mL reaction) and are in the process of obtaining kinetic parameters (k_{cat} and K_m) for comparison with the *in vivo*-produced enzyme. The steps involved in purification are gel filtration on Sephadex G-75, chromatofocusing on PRE 94 or Mono P FPLC, and anion exchange chromatography on Mono Q FPLC (see Appendix).

Supplementation of an *in vitro* reaction with [^{35}S]-methionine, followed by SDS polyacrylamide gel electrophoresis and autoradiography, results in a single band on a gel with a molecular weight corresponding to β-lactamase (29,000). If a plasmid is used in which the codon for serine 70 has been replace with the stop codon TAG, this band becomes very faint. This faint band does not increase in intensity if the reaction is supplemented with 5μg of non

weight corresponding to β-lactamase (29,000). If a plasmid is used in which the codon for serine 70 has been replace with the stop codon TAG, this band becomes very faint. This faint band does not increase in intensity if the reaction is supplemented with 5μg of non aminoacylated $tRNA_{CUA}$. If the reaction is supplemented with 5 μg of phenylanyl $tRNA_{CUA}$ (20% aminoacylated), however, a band of equal molecular weight and intensity to that produced by the wild-type plasmid is observed. These results suggest that the constructed suppressor tRNA is neither acylated nor deacylated *in vitro*, and it appears that the suppression efficiency of Phe-$tRNA_{CUA}$ is close to 100%. Furthermore, a reaction containing mutant plasmid and [^{14}C] Phe-$tRNA_{CUA}$, with no [^{35}S]-methionine added, results, after extensived autoradiography, in a band with molecular weight corresponding to β-lactamase.

The mutant gene in which phenylalanine at position 66 of RTEM β-lactamase has been replaced with the stop codon TAG should make it possible to observe restored enzymatic activity upon suppression of this mutant with Phe-$tRNA_{CUA}$, an experiment that is not currently possible with the serine 70 mutant. Catalytic parameters of the enzyme thus produced will be obtained and compared with *in vivo*-produced enzyme to confirm its identity. In addition, peptide mapping studies are underway which will make it possible to localize a site-specifically inserted radioactive amino acid to one particular region of the enzyme.

Summary of Results - Year 1

(1) Mutagenesis of the β-lactamase (ser 70 and phe 66) gene and construction of the appropriate expression vectors.

(2) Construction and characterization of the nonsense suppressor $tRNA^{Phe}_{CUA}$. Optimization of the aminoacylation of the suppressor with phenylalanine.

(3) Construction and sequencing of the suppressor $tRNA^{Gly}_{CUA}$ gene.

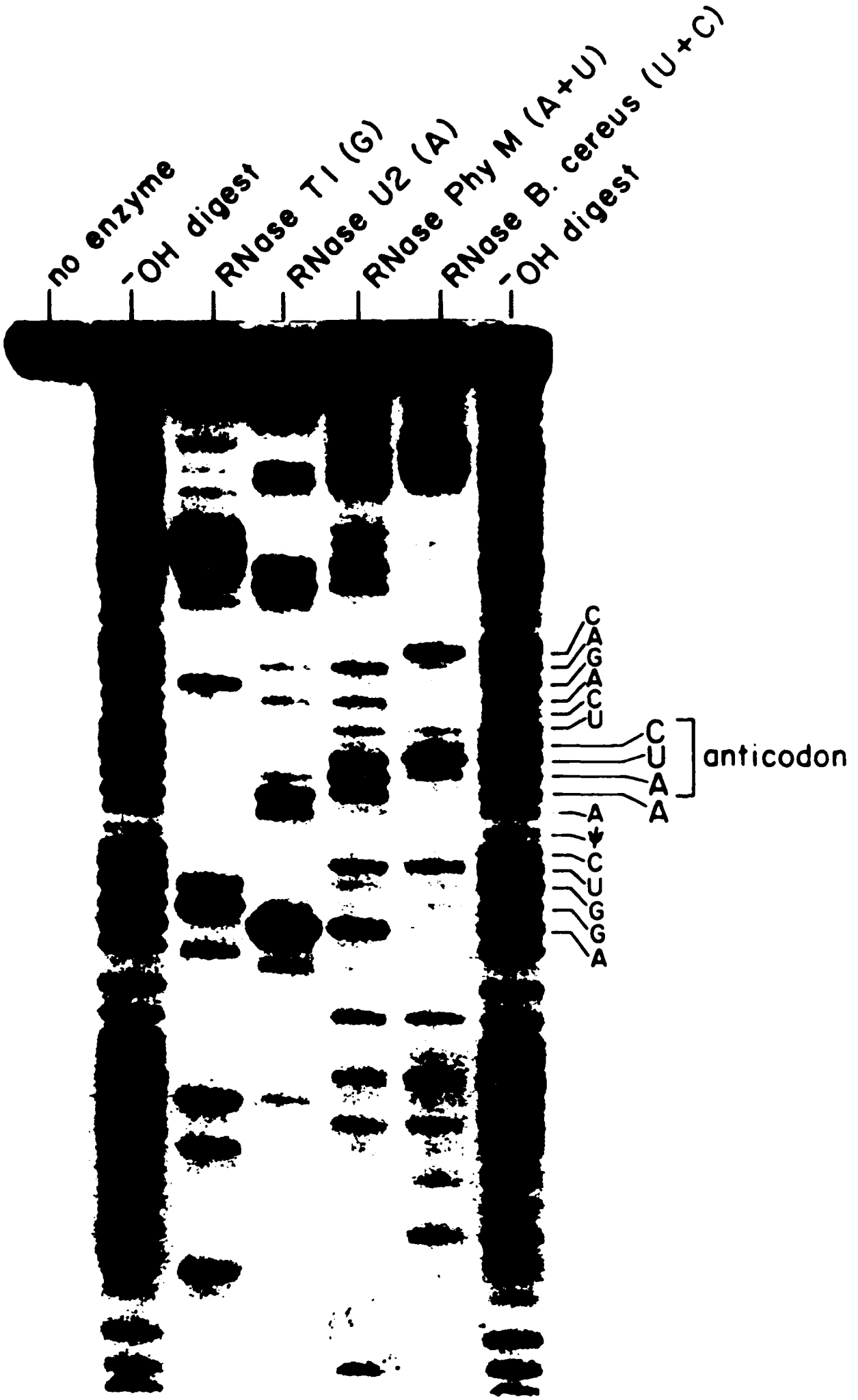
(4) Synthesis of large quantities of pCpA and progress toward the aminoacylation of pCpA with serine, phenylalanine and alanine.

(5) Expression of native β-lactamase in an efficient coupled transcription/translation system.

(6) Expression of β-lactamase containing ^{14}C-Phe 70 by suppression of the nonsense codon at position 70 of bla using ^{14}C-Phe-tRNA$_{CUA}$.

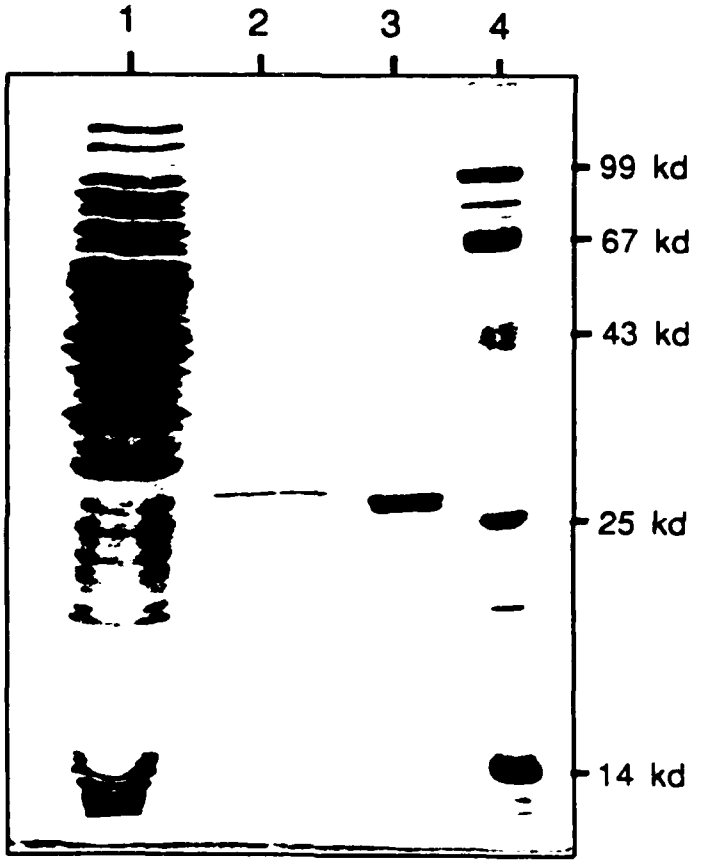
Appendix

A. Denaturing sequencing gel of suppressor, $tRNA_{CUA}$.


no enzyme
-OH digest
RNase T1 (G)
RNase U2 (A)
RNase Phy M (A+U)
RNase B. cereus (U+C)
-OH digest
CAGACU
CUAA
anticodon
A
CUGGA

B. Coomassie stained SDS-PAGE gel of *in vitro* produced β-lactamase. Lane 1, crude *in vitro* reaction; Lane 2, *in vitro* produced native β-lactamase with truncated leader sequence; Lane 3 *in vivo* produced native β-lactamase with truncated leader sequence; Lane 4, molecular weight standards.

PURIFICATION OF IN VITRO β-LACTAMASE



Purification Protocol

a) G-75 gel filtration. b) PBE 94 chromatofocusing
c) FPLC Mono Q anion exchange. d) G-75 gel filtration.

END

9-87

DTIC